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Haloacetonitrile Excretion as
Thiocyanate and Inhibition of
Dimethylnitrosamine Demethylase
A Proposed Metabolic Scheme

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**HALOACETONITRILE EXCRETION
AS THIOCYANATE AND INHIBITION
OF DIMETHYLNITROSAMINE DEMETHYLASE:
A PROPOSED METABOLIC SCHEME**

Michael A. Pereira, Luan-Ho C. Lin, Joan K. Mattox

U.S. Environmental Protection Agency, Health
Effects Research Laboratory, Cincinnati, Ohio

Haloacetonitriles, contaminants present in chlorinated drinking water, were administered orally to rats, and the urinary excretion of thiocyanate was measured as an index of cyanide release. The urinary excretion of thiocyanate accounted for 14.2% of the dose of monochloroacetonitrile; 7.7–12.8% of the dose of bromochloro-, dichloro-, and dibromoacetonitrile; and 2.25% of the dose of trichloroacetonitrile. The haloacetonitriles inhibited rat-liver microsomal dimethylnitrosamine (DMN) demethylase in an in vitro assay system. Dibromo- and bromochloroacetonitrile were the most potent inhibitors of DMN demethylase, with $K_i = 3-4 \times 10^{-5}$ M; dichloro- and trichloroacetonitrile were the next most potent, with $K_i = 2 \times 10^{-4}$ M; and monochloroacetonitrile was the least potent inhibitor, with $K_i = 9 \times 10^{-2}$ M. Trichloroacetonitrile, but not dibromoacetonitrile, when administered orally inhibited hepatic DMN demethylase activity. The relative capacity of the haloacetonitriles to inhibit DMN demethylase and to be excreted as thiocyanate did not correlate.

INTRODUCTION

Dihaloacetonitriles have been found in chlorinated drinking water at a concentration of 0.3–8.1 ppb, which was about 10% of the molar concentration of trihalomethanes (which can be as high as 100 ppm) (Oliver, 1983). During chlorination, algae, fulvic acid, and proteinaceous material in water are precursors for the dihaloacetonitriles (Oliver, 1983; Trehly and Beiber, 1981). We have previously reported that haloacetonitriles can bind to the nucleophilic trapping agent *p*-nitrobenzopyridine, to polyadenosine, and to DNA, and can produce strand breaks in the DNA of cultured human CCRF-CEM cells (Daniel et al., 1983). Therefore, the exposure of humans to haloacetonitriles via drinking water might represent a genotoxic and thus carcinogenic hazard. Aminoacetonitrile, a structural analog of haloacetonitriles, has been shown to inhibit dimethylnitrosamine (DMN) demethylase and diethylnitrosamine (DNA) deethylase; to inhibit the alkylation by DMN of liver RNA; and to inhibit the liver carcinogenicity and toxicity

Requests for reprints should be sent to Michael A. Pereira, U.S. Environmental Protection Agency, Health Effects Research Laboratory, 26 West St. Clair Street, Cincinnati, Ohio 45268.

of DENA (Fiume et al., 1970; Hadjiolov and Markov, 1973; Mundt and Hadjiolov, 1974). In this communication, we describe the propensity of haloacetonitriles to inhibit DMN demethylase activity and to be excreted as thiocyanate.

MATERIAL AND METHODS

Chemicals

DMN, dibromoacetonitrile (DBAN), and monochloroacetonitrile (MCAN) were purchased from Aldrich (Milwaukee, Wis.), dichloroacetonitrile (DCAN) and trichloroacetonitrile (TCAN) from Pfaltz and Bauer, Inc. (Stanford, Conn.), and propionitrile from Fluka Chemical Corporation (Hauppauge, N.Y.). The purity was determined by GC/MS to be 100% for MCAN, 100% for DCAN, 98% for TCAN (2% DCAN), and 96% for DBAN (4% tribromoacetonitrile). Bromochloroacetonitrile (BCAN) was synthesized in our laboratory by Dr. Paul Ringhand using a method suggested by Dr. I. I. Bieber (Florida Atlantic University). Briefly, the synthesis involved premixing equivalent concentrations of *N*-chloro- and *N*-bromosuccinimide and adding them to a chilled aqueous solution of cyanoacetic acid. Following filtration, the reaction mixture (filtrate) was extracted with methylene chloride and dried over anhydrous sodium sulfate. The resulting extract was purified by fractional vacuum distillation. The structure of the purified material was confirmed by mass spectrometry. The synthesized BCAN contained approximately 4% DBAN and 3% DCAN.

Animals

Male Sprague-Dawley rats purchased from Charles River (Portage, Mich.) were used throughout these studies. DMN demethylase studies were carried out on rats weighing 80–100 g, since young rats possess a high level of enzyme activity (Davis et al., 1976). Rats weighing 300–375 g were used in determination of the urinary excretion of thiocyanate, as described by Silver et al. (1982). The animals received Purina Rodent Chow (Ralston Purina Company, St. Louis, Mo.) and distilled water *ad libitum*.

Preparation of Microsomes

All animals were fasted overnight and sacrificed by decapitation. Microsomes were prepared by homogenizing the liver in 4 parts by volume of 0.05 M potassium phosphate buffer, pH 7.4, containing 1.15% potassium chloride. The homogenate was centrifuged sequentially at 12,000 × g for 30 min and 105,000 × g for 75 min at 4°C. The resulting pellet was resuspended in the same buffer to give a suspension containing 10–14 mg protein/ml. The protein concentration was measured by the procedure of Lowry et al. (1951).

Determination of DMN Demethylase Activity

DMN demethylase activity was assayed in an incubation mixture that contained 5–7 mg microsomal protein, magnesium chloride (20 mM), semicarbazide (9 mM), an NADPH regenerating system consisting of glucose 6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (3 units) and NADP (0.8 mM), DMN (80 mM or other concentrations), and phosphate buffer, pH 7.4 (50 mM), in a total volume of 4 ml. In some incubations, various concentrations of the different haloacetonitriles were also present. The reaction was started immediately after the addition of the haloacetonitrile by the addition of the substrate, and the samples were incubated at 37°C aerobically. After 30 min the reaction was stopped by the addition of 1 ml of 15% zinc sulfate and 1 ml saturated barium hydroxide, while the reaction mixture was rapidly cooled in an ice-water bath. In the control incubation, the substrate was added after the protein was precipitated. The protein-free supernatant fraction was assayed by a modified Nash procedure specific for formaldehyde (Cochin and Axelrod, 1959).

Estimation of Thiocyanate in Urine

The urine was collected from individual rats for 24 h before and after the administration by gavage, at 0.75 mmol/kg, of various haloacetonitriles dissolved in tricapylin; the urine was filtered before analysis of the thiocyanate content. The 0.75-mmol/kg dose was chosen for comparison to Silver et al. (1982), who used this dose to compare 10 other nitriles, and because it represents 10–30% LD50 of the haloacetonitriles. Thiocyanate was determined according to the procedure of Contessa and Santi (1973). Samples of 0.2 ml urine were diluted to 4 ml with water, and 1 ml ferric nitrate solution [5 g $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ in 5 ml concentrated nitric acid, made up to 100 ml] was added. The absorbance was measured at 450 nm against a urine blank (0.2 ml urine diluted to 5 ml with water). The percentage of the dose excreted as thiocyanate in 24 h was calculated after subtraction of the endogenous thiocyanate determined from the 24-h urine sample obtained immediately prior to the administration of the haloacetonitrile. The percent of an oral dose of 0.75 mmol propionitrile/kg excreted in 24 h was used as a positive control and was similar to the 65% of dose found by Silver et al. (1982).

RESULTS

Inhibition of DMN Demethylase Activity

The ability of DBAN to inhibit microsomal DMN demethylase activity is presented in Fig. 1. Two forms of rat-liver DMN demethylase activity were demonstrated in the double-reciprocal plots: one with a K_m of 2×10^{-5} , and the other with a K_m of 7×10^{-2} M. Our results are consistent

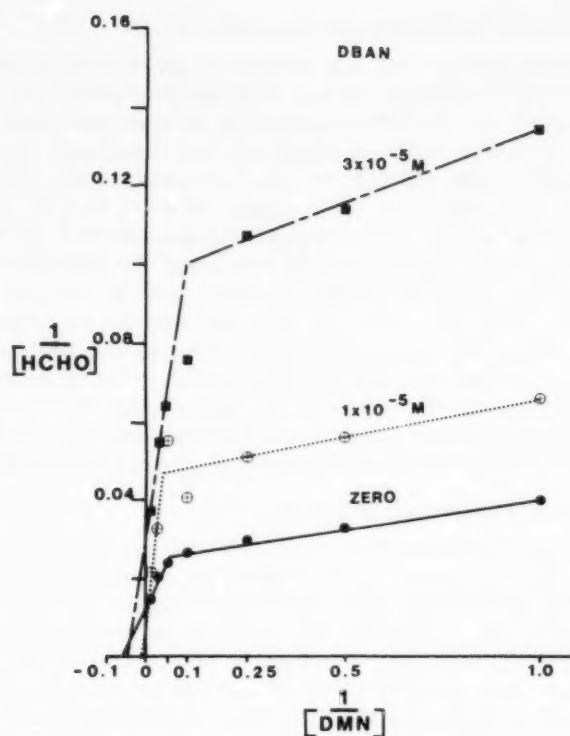


FIGURE 1. Double-reciprocal plots of dimethylnitrosamine concentration versus DMN demethylase activity. The activity was assayed in the presence or absence of 1×10^{-5} or $3 \times 10^{-5} M$ DBAN. The incubation medium contained 5.4 mg microsomal protein in a total volume of 4 ml. The incubation was performed at $37^\circ C$ and for 30 min. Enzymatic activity is expressed as μmol formaldehyde (HCHO) produced/mg protein in 30 min.

with those reported by Tu and Yang (1983) for the low- K_m ($7 \times 10^{-5} M$) and high- K_m ($3.9 \times 10^{-2} M$) forms of rat liver microsomal DMN demethylase. The inhibition by DBAN was not competitive but was either noncompetitive or uncompetitive. Figure 2 presents the dose-response relationship of the inhibition by haloacetonitriles of DMN demethylase activity. DBAN and BCAN were the most potent inhibitors, with $K_i = 3-4 \times 10^{-5} M$; DCAN and TCAN were the next most potent inhibitors with $K_i = 2 \times 10^{-4} M$; and MCAN was the least potent inhibitor, with $K_i = 9 \times 10^{-2} M$. The effect of *in vivo* administration of DBAN or TCAN on DMN demethylase activity is presented in Table 1. DBAN (0.75 mmol/kg) or TCAN (0.75 mmol/kg) were administered by gavage, and the liver microsomes were isolated 3 or 18 h later, which corresponds to the time of maximum macromolecular binding by direct and indirect binding agents.

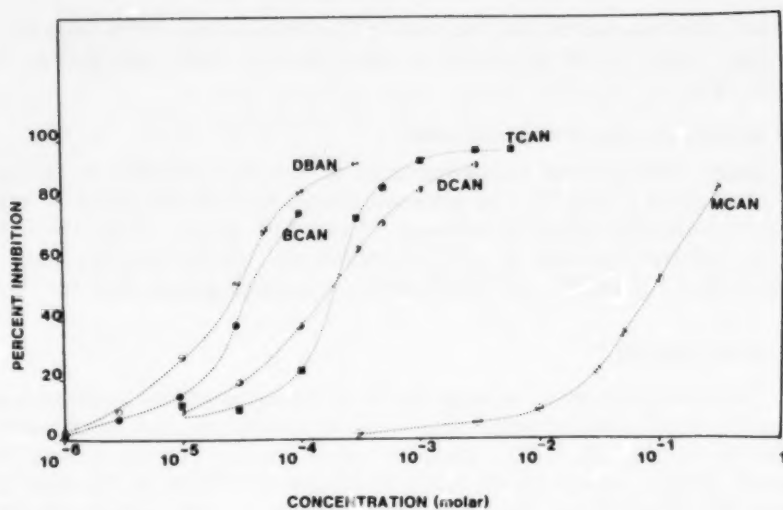


FIGURE 2. Dose-response relationships of inhibition by haloacetonitriles of DMN demethylase. The incubations contained 5.5–6.5 mg microsomal protein and 80 mM DMN. The incubation was performed at 37°C for 30 min, and the percent inhibition of the enzymatic activity, expressed as μmol formaldehyde formed/mg protein in 30 min, is presented.

TABLE 1. Effects of DBAN on Rat-Liver DMN Demethylase Activity^a

Chemical	Demethylase activity ^b	
	3 h	18 h
Experiment 1		
DBAN	90.2 $\pm$ 3.9 (102) ^c	89.3 $\pm$ 5.4 (128) ^d
Tricaprylin	88.0 $\pm$ 4.3	69.6 $\pm$ 1.9
Experiment 2		
TCAN	48.1 $\pm$ 2.7 (71) ^e	47.3 $\pm$ 3.1 (65) ^e
Tricaprylin	67.7 $\pm$ 3.8	72.9 $\pm$ 1.6

^aMale Sprague-Dawley rats were administered 0.75 mmol DBAN/kg by gavage, and dimethylnitrosamine demethylase activity was measured in isolated microsomes.

^bDMN demethylase activity, determined as μmmol HCHO/mg protein $\cdot$ 30 min, is mean $\pm$ SE for 6 rats.

^cResults in parentheses are percentage of control.

^dDifferent from control by Student's *t*-test, $p < 0.01$.

^eDifferent from control by Student's *t*-test, $p < 0.001$.

DBAN administered to rats did not inhibit microsomal DMN demethylase activity, while TCAN produced an approximately 30% inhibition at both 3 and 18 h.

Urinary Excretion of Thiocyanate

Orally administered haloacetonitrile to rats was excreted in the urine as thiocyanate (Table 2). The haloacetonitriles were administered at a dose of 0.75 mmol/kg, which is between 10 and 33% of the LD50. The extent of the urinary excretion in 24 h of MCAN and DBAN as thiocyanate was greater than for DCAN and DBAN, which was much greater than for TCAN.

DISCUSSION

The haloacetonitriles were excreted in the urine of rats as thiocyanate. Figure 3 depicts the possible metabolism of mono-, di-, and trihaloacetonitriles to cyanide. A similar metabolic pathway for cyanide release from organic nitriles, which results in the formation of aldehydes, has been proposed by Silver et al. (1982). It is proposed that monohaloacetonitrile yields formaldehyde, dihaloacetonitrile yields formyl cyanide or formyl halide, and trichloroacetonitrile yields phosgene or cyanofarmyl chloride. Mono- and dichloroacetonitriles might be metabolized by mixed-function oxidase to yield cyanochloromethanol and cyanodichloromethanol, respectively. The proposed products of the release of cyanide from haloacetonitriles are direct-acting alkylating agents, highly toxic chemicals, and, at least in the case of formaldehyde, a carcinogen (Svenberg et al., 1980). The released cyanide is further metabolized by rhodanese (EC 2.8.1.1) to thiocyanate. Our results of the urinary excretion of thiocyanate and the *in vivo* inhibition by TCAN of DMN demethylase activity would indicate a systemic absorption of haloacetonitriles and their conversion to highly toxic metabolites.

TABLE 2. Excretion of Halogenated Acetonitriles as Thiocyanates^a

Haloacetonitrile	% of dose
MCAN	14.2 ± 3.8
BCAN	12.8 ± 1.5
DCAN	9.28 ± 1.09
DBAN	7.67 ± 2.13
TCAN	2.25 ± 0.82
Propionitrile	42.0 ± 6.2

^aThe haloacetonitriles were administered by gavage as 0.75 mmol/kg body weight in tri-caprylin to male Sprague-Dawley rats, and a 24-h urine sample was obtained. Results are mean ± SE for 5-6 rats.

to break strands of DNA. The discrepancy between the alkylation potential and the extent of metabolism to thiocyanate of MCAN and TCAN and their ability to inhibit DMN demethylase and to break DNA could result from the much greater chemical reactivity of the proposed TCAN metabolites, phosgene and cyanoformyl chloride, compared to the proposed MCAN metabolite, formaldehyde (Fig. 3). Therefore, even though TCAN breaks down to cyanide to a much lesser extent than MCAN, the phosgene and cyanoformyl chloride formed when TCAN does undergo metabolism would be expected to be more potent as inhibitors of DMN demethylase and as DNA strand-breakers than formaldehyde. Thus, the relative biologic activity of the haloacetonitriles would be determined by the activity of both the parent chemicals and their metabolites. It is also possible that, compared to TCAN, the higher reactivity (alkylation potential) of MCAN could indicate that MCAN is too reactive to reach target intracellular macromolecules.

Haloacetonitriles—especially DCAN, BCAN, and DBAN—have been found in drinking water at concentrations of 0.3–8.1 ppb (Oliver, 1983). At higher oral doses of haloacetonitriles, uptake and metabolism was demonstrated by excretion of thiocyanate and by inhibition by TCAN of hepatic DMN demethylase activity. The haloacetonitriles are direct-acting alkylating agents (Daniel et al., 1983) that might result in liver and other organ toxicity and carcinogenesis. Dose-response studies are necessary before extrapolation can be performed from these high dose effects to estimation of any toxicity from the haloacetonitriles at the low level found in drinking water. The metabolism of haloacetonitrile and the inhibition of hepatic DMN demethylase demonstrate the need for these dose-response studies and the need for more sensitive procedures to detect these effects at the low dose level that results from exposure from drinking water.

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